(2)

AD-A197 721

OCUMENTATION PAGE

Form Approved OMB No. 0704-0188

...SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHO...	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution unlimited - approved for public release.
2b. DECLASSIFICATION/DOWNGRADI...CHEDULE	
4. PERFORMING ORGANIZATION REP...UMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: U.S. Army Medical Research Institute of Infectious Diseases; 6b. OFFICE SYMBOL (if applicable): SGRD-UIS-F	7a. NAME OF MONITORING ORGANIZATION: U.S. Army Medical Research and Development Command
6c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5011	7b. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012
8a. NAME OF FUNDING/SPONSORING ORGANIZATION; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)

Are Reactive Oxygen Species Involved in Microcystin-LR Intoxication?

12. PERSONAL AUTHOR(S)

David R. Franz, Ross D. LeClaire, Wade B. Lawrence, and David L. Bunner

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Interim	FROM _____ TO _____	12 May 1988	17

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	microcystin, reactive oxygen species, mice cyanoginosin

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Because reactive oxygen species are formed during the metabolism of several toxins that cause similar pathologic changes, we hypothesized that compounds that alter the concentration of reactive oxygen species would alter the toxic effects of the peptide-hepatotoxin produced by Microcystis aeruginosa. We show here that pretreatment with a free radical generator, alloxan; a free radical scavanger, butylated hydroxyanisole; or an iron chelator/inhibitor of lipid peroxidation, desferrioxamine, did not alter the severity of microcystin-LR intoxication in fed mice. Furthermore, fasting mice for 24 hr before testing, which unmasks lipid peroxidation in paracetamol intoxication, did not alter the effect of BHA pretreatment.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) / 22c. OFFICE SYMBOL

To Tom
13 May 93

ARE REACTIVE OXYGEN SPECIES INVOLVED IN MICROCYSTIN-LR INTOXICATION?

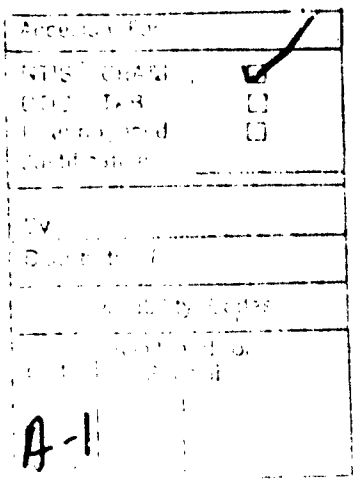
DAVID R. FRANZ, ROSS D. LECLAIRE, WADE B. LAWRENCE, AND DAVID L. BUNNER

Pathophysiology and Pathology Divisions, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA 21701-5011

RUNNING TITLE: Microcystin and Reactive Oxygen Species

SEND PROOFS TO: LtC David R. Franz
Pathophysiology Division
U.S.A.M.R.I.I.D.
Fort Detrick, Frederick, MD 21701-5011

D. R. Franz, R. D. LeClaire, W. B. Lawrence, and D. L. Bunner. Are Reactive Oxygen Species Involved In Microcystin-LR Intoxication? Toxicon xx,xxx-xxx,19xx.--Because reactive oxygen species are formed during the metabolism of several toxins that cause similar pathologic changes, we hypothesized that compounds that alter the concentration of reactive oxygen species would alter the toxic effects of the peptide-hepatotoxin produced by Microcystis aeruginosa. We show here that pretreatment with a free radical generator, alloxan; a free radical scavanger, butylated hydroxyanisole; or an iron chelator/inhibitor of lipid peroxidation, desferrioxamine, did not alter the severity of microcystin-LR intoxication in fed mice. Furthermore, fasting mice for 24 hr before testing, which unmasks lipid peroxidation in paracetamol intoxication, did not alter the effect of BHA pretreatment.



INTRODUCTION

The toxic cyclic heptapeptide, microcystin-LR (cyanoginosin-LR, microcystin-A), isolated from the cyanobacterium Microcystis aeruginosa, kills mice within 1 hr after i.v. or i.p. exposure to an acute lethal dose. Hepatic engorgement and thrombocytopenia develop 30-40 min postchallenge. Histologic findings after death include severe hemorrhagic liver necrosis, renal nephrosis, and microscopic pulmonary thrombi (ADAMS et al., 1985). The hepatic lesion is in the centrilobular region (CARMICHAEL, 1986), where drug metabolizing enzymes (eg. microsomal P-450) are present in highest concentrations (SHERLOCK, 1986), suggesting pathologic changes might be mediated by a microcystin metabolite rather than directly by the toxin. Free radical formation occurs during the metabolism of several hepatotoxins that cause similar lesions (FARBER AND GERSON, 1984), therefore, we hypothesized that compounds that alter the concentration of reactive oxygen species would also alter the toxic effects of microcystin.

Alloxan, a free radical generator, causes hemolysis of tocopherol-deficient erythrocytes, damages islet cells, and cures Plasmodium vinckei-infected mice, all thought to be mediated by

reactive oxygen species (FEE and TEITELBAUM, 1972; MALAISSE, 1982; CLARK AND HUNT, 1983). Desferrioxamine (DFA), an iron chelator that inhibits lipid peroxidation and the formation of hydroxyl radicals from superoxide and hydrogen peroxide (GUTTERIDGE et al., 1979), blocks the above listed effects of alloxan (CLARK AND HUNT, 1983). Butylated hydroxyanisole (BHA), a free radical scavenger, protects mice against alloxan-induced diabetes (COWDEN et al., 1985). These three compounds were studied in combination with microcystin to test the stated hypothesis.

MATERIALS AND METHODS

Four groups of 28, fed (NIH-07 diet), Charles River, male, CD-1 outbred, VAF/plus mice, weighing 20-25 g, were pretreated with either alloxan, BHA, DFA, or saline. Alloxan was administered i.v. at a dose of 50 mg/kg (12.5 mg/ml) 30 min before challenge. Butylated hydroxyanisole was given i.p., 120 mg/kg (30 mg/ml in pure olive oil) 1 hr before challenge (COWDEN et al., 1985). Desferrioxamine was given i.p., 200 mg/kg (100mg/ml) 30 min before challenge (CLARK AND HUNT, 1983). Physiologic saline was

given i.p., 4 ml/kg, 30 min before challenge. Within the four test groups, seven sets of four animals each were given one of six doses of microcystin (30, 45, 68, 101, 152, and 228 ug/kg) or physiologic saline s.c.

In a companion study, two groups of 18 mice were fed and two groups were fasted for 24 hr. One hr before intoxication, one fed group and one fasted group were treated (i.p.) with BHA, 120mg/kg. Within the four test groups, seven sets of three animals each were given one of six doses of microcystin (30-228 ug/kg) or physiologic saline s.c.

The purity of microcystin (source: W.W. Carmichael, Wright State Univ., Dayton, OH) was >95% as measured by high-pressure liquid chromatography and thin-layer chromatography. Mice surviving toxin challenge were killed 4 hr postchallenge by exposure to carbon monoxide. All animals were necropsied; livers were weighed and, in the first study, immersion-fixed in buffered 10% formalin. Median lethal doses (LD_{50}s) were calculated by a moving averages method (WEIL, 1952). Liver weights and time to death were analyzed by ANOVA and Tukey's HSD.

RESULTS

The LD_{50} of microcystin was not altered by pretreatment with alloxan, BHA, or DFA (Table 1) . None of the three drugs altered liver weight (Fig. 1). With the exception of BHA in the 228 ug/kg group, survival time (Fig. 2) was not changed by pretreatment with the test compounds.

Liver lesions, typically centrilobular hepatocellular coagulative necrosis, degeneration, hemorrhage, and congestion, were less severe in mice pretreated with BHA prior to receiving 45 ug/kg microcystin than with the other compounds tested. At 68 ug/kg microcystin, there was no discernible difference between treatment groups.

Fasting did not alter the effect of BHA pretreatment of intoxicated animals (liver weight and time to death data not shown), though fasting appeared to enhance the toxicity of microcystin (Table 2).

DISCUSSION

The postmortem pathologic changes after microcystin intoxication have been described, but the cellular events leading to death is not known. ADAMS et al. (1985) demonstrated that sublethal hepatocellular damage induced by carbon tetrachloride before toxin challenge prevents acute death, apparently by disrupting hepatic enzymes; this finding supports the microcystin metabolite hypothesis. If the mixed function-oxidase enzymes do alter the parent toxin, a number of possibilities exist. Reactive metabolites produced within the hepatocyte could covalently bind to intracellular membrane lipids or proteins or initiate lipid peroxidation, resulting in the formation of other toxic products. Enzymatic metabolism of the toxin could result in the formation of electrophilic metabolites that, through macromolecular binding, can cause death of hepatocytes, though this might not be expected to occur in the 30-40 min before death in our model. Finally, hepatocyte injury could occur through the production of activated oxygen species (FARBER AND GERSON, 1984). Though we have not explained the reduction in pathologic change in the BHA-45 ug/kg group or the slight increase in time to death in the BHA-228 ug/kg group, our data suggest that neither reactive oxygen species nor lipid peroxidation products alone

play a major, causal role in the pathogenesis of mycrocystin intoxication in mice.

It has been shown that paracetamol induces dose-dependant lipid peroxidation in starved, but not in fed mice (WENDEL et al., 1979). This fact, and the trends suggesting some protection by BHA, led us to repeat the BHA studies in fasted animals, reasoning that, as in the case of paracetamol, fasting might unmask free radical-induced lipid peroxidation which could be altered by BHA pretreatment. The effect was not seen in our model.

Increased toxicity of mycrocystin in fasted rats has been previously noted (MIURA et al., The FASEB Journal,2, A1351, Abstract #6134). Strubelt et al. (1981) demonstrated that fasting before intoxication markedly increases marker hepatic enzyme levels in mice challenged with carbon tetrachloride, thioacetamide, paracentamol, or bromobenzene, all toxins known to be metabolized in the liver to toxic intermediates. They also reported that fasting mice for 24 hr decreased liver glycogen by 93% and hepatic GSH by 50% while increasing liver triglycerides by 162%. Cytochrome P-450 content was decreased by one-third while hydroxylation of aniline and N-demethylation of aminopyrine were both increased in liver homogenates from fasted mice. A

number of these factors could be involved in the pathogenesis of microcystin intoxication.

RUNNEGAR et al. (1987) have recently demonstrated that, *in vitro*, microcystin causes a time- and dose-dependent decrease in hepatocyte GSH levels; however, blebbing of the hepatocytes preceeds any large decrease in GSH levels. The dynamics of GSH metabolism and its role in limiting pathologic change are not clearly defined. In the case of other hepatotoxins, equivalent depletion of GSH (by fasting or diethyl maleate treatment) results in varying alterations of hepatotoxicity (REED AND FARISS, 1984). Though the role of phase II biotransformation reactions in limiting microcystin-induced cell injury remains to be established, these data suggest that reactive oxygen species do not play a major role in the pathogenesis of intoxication.

REFERENCES

Adams, W.H., Stoner, R.D., Adams, D.G., Slatkin, D.N. and Seigelman, H.W. (1985) Pathophysiologic effects of a toxic peptide from Microcystis aeruginosa. Toxicon **23**, 441.

Carmichael, W.W. (1986) Algal toxins. Adv. Botanic. Res. **12**, 47.

Clark, I.A. and Hunt, N.H. (1983) Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. Infect. Immun. **39**, 1.

Cowden, W.B., Lewis-Hughes, P.H. and Clark, I.A. (1985) Protection against alloxan-induced diabetes in mice by the free radical scavenger butylated hydroxyanisole. Biochem. Pharmac. **34**, 3601.

Farber, J.L. and Gerson, R.J. (1984) Mechanisms of cell injury with hepatotoxic chemicals. Pharmacol. Rev. **36**, 71S.

Fee, J.A. and Teitelbaum, H.D. (1972) Evidence that superoxide dismutase plays a role in protecting red blood cells against

peroxidative hemolysis. Biochem. Biophys. Res. Comm. **49**, 150.

Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1979) Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. Biochem. J. **184**, 469.

Malaisse, W.J. (1982) Alloxan toxicity to the pancreatic B-cell: a new hypothesis. Biochem. Pharmac. **31**, 3527.

Reed D.J. and Fariss, M.W. (1984) Glutathione depletion and susceptibility. Pharmac. Rev. **36**, 25S.

Runnegar, M.T.C., Andrews, J. Gerdes, R.G. and Falconer, I.R. (1987) Injury to hepatocytes induced by a peptide toxin from the cyanobacterium Microcystis aeruginosa. Toxicon. **25**, 1235.

Sherlock, S. (1986) The spectrum of hepatotoxicity due to drugs. Lancet **2**, 440.

Strubelt, O., "Dost-Kempf, E., Siegers, C-P., Younes, M., Volpel, M., Preuss, U., and Dreckmann, J.G. (1981) The influence of

fasting on the susceptibility of mice to hepatotoxic injury. Toxicol. Appl. Pharmacol. **60**, 66.

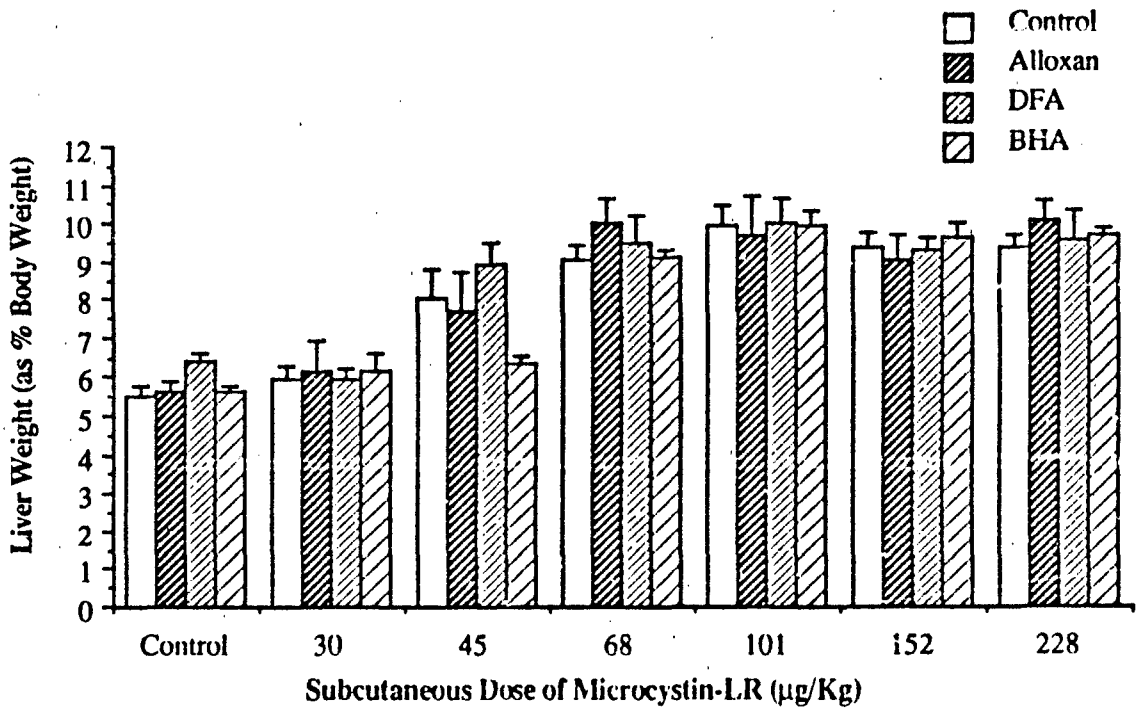
Weil, C.S. (1952) Tables for convenient calculation of median-effective dose (LD50 or ED50) and instructions in their use. Biometrics **8**, 249.

Wendel, A., Feuerstein, S., and Konz, K-H. (1979) Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. Biochem. Pharmacol. **28**, 2051.

LEGENDS FOR FIGURES

Fig 1. Liver weight, as percent body weight, of mice that died before, or were killed at, 4 hr after subcutaneous challenge with the dose of microcystin-LR stated. Before challenge, animals were pretreated with either saline, alloxan, DFA, or BHA. Values presented as means $\pm$ S.E. No significant difference at $p < 0.05$. (n = 4)

Fig 2. Survival time in hr for mice from groups in which all animals died within 4 hr after challenge with the dose of microcystin-LR stated. Before challenge, animals were pretreated with either saline, alloxan, DFA, or BHA. Values presented as means $\pm$ S.E. * $p < 0.05$. (n = 4)


Control
Alloxan
DFA
BHA
Liver Weight (as % Body Weight)
0
1
2
3
4
5
6
7
8
9
10
11
12
Control
30
45
68
101
152
228
Subcutaneous Dose of Microcystin-LR (µg/Kg)

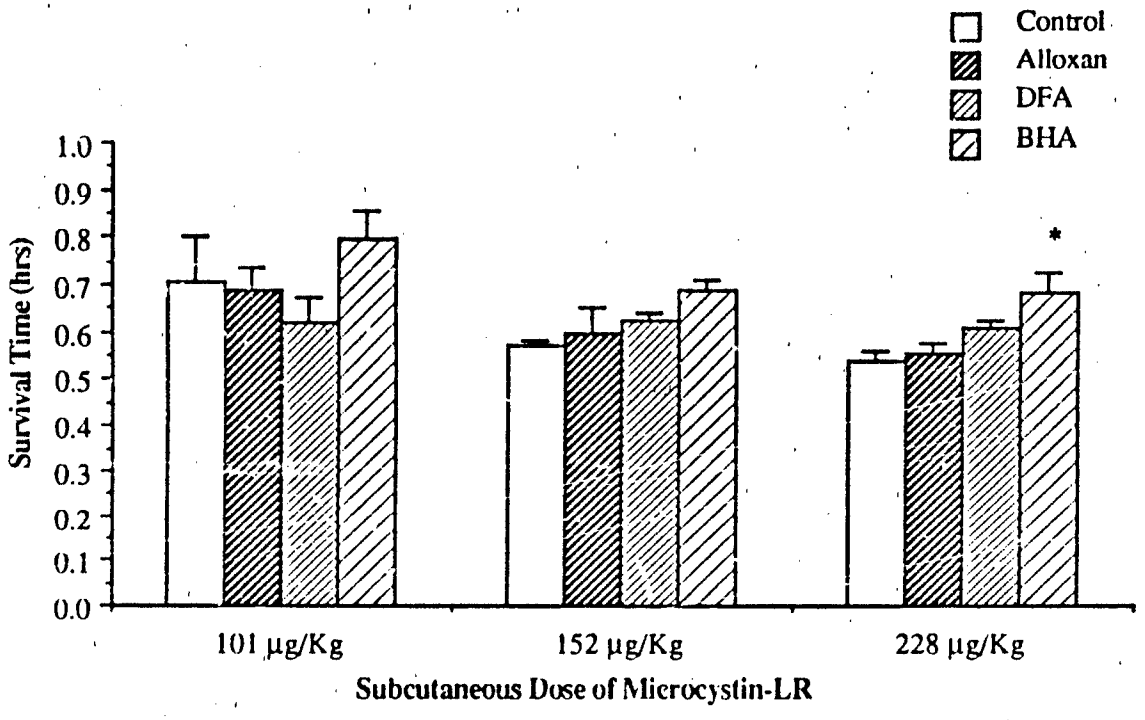

Control
Alloxan
DFA
BHA
Survival Time (hrs)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
101 µg/Kg
152 µg/Kg
228 µg/Kg
Subcutaneous Dose of Microcystin-LR

TABLE 1. EFFECT OF MICROCYSTIN-LR ON LD_{50} VALUES AFTER BHA PRETREATMENT IN FED MICE AND MICE FASTED FOR 24 HR.

Pretreatment	LD_{50} (4 hr)‡ (μg/Kg)	95% confidence interval (μg/Kg)
Saline	45.0	35.6 - 56.8
Alloxan (50 mg/Kg)	55.1	41.4 - 73.4
BHA (120 mg/Kg)	55.1	-
DFA (200 mg/Kg)	49.8	36.5 - 67.9

‡ n = 4 mice per group.

TABLE 2. EFFECT OF PRETREATMENT ON LD_{50} VALUES OF MICROCYSTIN-LR IN FED MICE.

Pretreatment	LD_{50} (4 hr)‡ (µg/Kg)	95% confidence interval (µg/Kg)
Fed, Saline	55.1	-
Fed, BHA	48.2	30.2 - 76.9
Fasted, Saline	32.1	20.1 - 51.3
Fasted, BHA	32.1	20.1 - 51.3

‡ n = 3 mice per group.